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13. ABSTRACT (Maximum 200 Words) Control of the activation of apoptosis is important in protection against cancer. PML oncogenic domains (PODs) are subnuclear macromolecular complexes that play a critical role in the regulation of apoptosis. Daxx and PML, two pro-apoptotic proteins are found in PODs, along with at least a dozen or so other proteins. The mechanism by which PODs modulate apoptosis is poorly understood. Here we examine the localization of various components of the apoptosis pathway and find co-localization of certain key pro-apoptotic proteins in the PODs. This work suggests a model on how PODs may initiate apoptosis, and may help identify new approaches to modulate apoptosis for cancer therapy.				
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INTRODUCTION

Apoptosis is a physiological process of cell auto-destruction that plays a critical role in the protection against cancer. We previously identified a pro-apoptotic protein Daxx (1). Daxx can be found in large amount in the promyelocytic leukemia protein (PML) oncogenic domains (PODs) (also referred to as nuclear domain 10 or ND10, PML nuclear bodies or PML-NB, and Kremer bodies), which are nuclear macromolecular complexes that regulate multiple cellular processes including cell growth, anti-viral defense, and apoptosis (2, 3). Disruption of PODs is associated with tumorigenesis. In most cases of acute promyelocytic leukemia (APL), for example, these speckled-shaped structures are dispersed into aberrant microspeckles due to the t(15;17) chromosomal translocation that fuses the eponymous component of PODs, PML, to retinoic acid receptor α (RAR α). In addition, PODs also regulate the function of p53, a prominent tumor suppressor protein. Mutations in p53 are found in over 50% of human tumors including breast cancer. The mechanisms by which PODs promote apoptosis and prevent tumorigenesis remain poorly understood. Caspases, a group of aspartic acid-specific cysteine proteases, are the key mediators of apoptosis and these proteases are involved in both the initiation and execution of cell death (4). During apoptosis, initiator caspases become activated through adapter-mediated oligomerization (5). Once activated, these caspases cleave and activate executioner caspases, which in turn cleave various cellular proteins, leading eventually to cell demise. To understand how PODs modulate apoptosis, we set out to examine their association with initiator caspases.

BODY

Result:

Subcellular Localization of human apoptosis caspases

To examine whether any caspase is localized at the PODs, we made green fluorescent protein (GFP) fusions of eight human caspases, and examined their subcellular localization.

These caspases included four apoptosis initiators (caspase-2, 8, 9, and -10), three apoptosis executioners (caspase-3, -6, and -7), and one putative inflammatory caspase (caspase-4). Because overexpression of wild type caspases often leads to rapid cell death, we either expressed the protease inactivate mutants of these caspases or co-expressed the wild type caspases with a pan-caspase inhibitor p35 to facilitate the detection of fluorescent signals. When transiently expressed in HeLa cells, caspase-3, -4, -8, and -10 mainly exhibited cytosolic localization while caspase-6, -7, and -9 displayed diffuse distribution in both the cytosol and the nucleus (Fig. 1). Notably, GFP-caspase-2 showed a pattern of discrete and interconnecting nuclear filaments in some cells and a pattern of nuclear dots resembling PODs in the others, consistent with a previous report on murine caspase-2 (6).

Co-localization of caspase-2 and caspase-8 with the PODs

Because the nuclear-dot localization pattern of caspase-2 closely resembled those of PODs, we immunostained the caspase-2-transfected cells with an antibody against PML. As shown in Figure 2, caspase-2 co-localized with the PODs. Similar colocalization was observed when 293 cells were used for transfection (Fig. 2C) or when a different anti-PML antibody was used (data not shown).

The POD localization of caspase-2 requires both the prodomain and protease domain but is independent of its adapter protein RAIDD.

To determine which region(s) of caspase 2 mediate their localization in the PODs, we fused the N-terminal prodomains and C-terminal protease domains to GFP. The C-terminus of caspase-2 acquired a cytoplasmic localization pattern, and its N-terminus, containing a caspase recruitment domain (CARD), resided in nucleus but not in any dot-like structures. These results showed that although the nuclear localization sequence of caspase 2 is within the pro-domain, targeting to PODs requires both the prodomain and the protease domain.

The adapter protein for caspase-2 is thought to be RAIDD, a bipartite adapter with a CARD domain and a DD domain (7). To determine whether the POD localization of caspase-2 is dependent on RAIDD, we examined the localization of RAIDD. RAIDD was located diffusely throughout the cells and was not detected in nuclear dots. Deletion analyses further showed that none of the constituent domains RAIDD were localized to the PODs. These results showed that the association of caspase-2 with the PODs is independent of its adapter protein RAIDD.

Because caspase-2 is normally present in the nucleus, we sought to examine whether endogenous caspase-2 is associated with the PODs. We immunostained live and dying cells with multiple antibodies against caspase-2, but these antibodies did not detect either caspase-2 or caspase-8 in PODs (data not shown). Previous immunoelectron microscopy analysis revealed a doughnut-like shape of PODs with a central core surrounded by a dense fibrillar ring that contains PML. To test the possibility that the caspase-2 in the PODs might not be accessible by the antibodies, we immunostained the caspase-2-GFP and caspase-8-GFP transfected cells using antibodies against the corresponding caspases. While these antibodies readily detected the GFP-caspase- fusion proteins present in the other parts of the cells, they could not spot those in the PODs (Fig. 3C).

In summary, we have shown in this study an initiator caspases, caspase-2, can associate with the PODs. Our work identifies a nuclear pathway that might directly regulate the activation of caspase-2 and reveals a possible connection between Daxx, PODs and apoptosis initiation.

Material and Methods:

Cell lines and Reagents

The human cervix carcinoma cell line HeLa and human embryonic kidney cell line 293T were obtained from ATCC and were cultured in DMEM with 10% FBS. A polyclonal anti-PML antibody was a gift from Dr. K.S. Chang (8). The following reagents were purchased from the indicated sources: z-VAD and Ac-IETD-AFC (Enzyme System Products); anti-PML mAb PG-M3 and rabbit anti-caspase-2 antibody H19 (Santa Cruz); and Texas red-conjugated anti-rabbit and anti-mouse IgG antibodies (Vector Lab).

Plasmids

The expression plasmid for PML (isoform IV with 633 amino acids in length, (2)) was made in pRK5. The following proteins or domains were C-terminally tagged with GFP in pEGFP-N3 (CLONTECH): Caspase-2, -3, -4, -8, -9, and -10, the COOH terminus of caspase-2 was NH₂-terminally tagged with GFP in pEGFP-C1 (CLONTECH). Caspase-2N-GFP was made by digesting caspase-2-GFP with BamHI plus SalI and religating the resulted fragment. Caspase-6 and -7 were C-terminally tagged with hrGFP (Invitrogene) in pRK5-C-hrGFP, while RAIDD, RAIDD-CARD (aa 1-104), and RAIDD-DD (aa 93-199) were N-terminally fused to and pRK5-N-hrGFP. The active site cysteine-to-serine (C/S) mutations of the caspases were generated by overlapping PCR.

Immunofluorescence

HeLa or 293 cells cultured in 24-well plates (with coverslips) were transfected with plasmids expressing various caspase-GFP fusions. 24 hr after transfection, the cells were fixed with 1% paraformaldehyde and permeablized in PBS/0.2% Triton X-100. Cells were then incubated for 1 hr with antibodies for PML or caspase-2, followed by 1 hr incubation with Texas red-conjugated secondary antibodies. Afterwards, the cells were dehydrated with

ethanol and mounted with Vectashield (containing DAPI, Vector Lab). The fluorescence signals were visualized with an epi-fluorescence microscope and image was recorded and analyzed using Phase3 program (Media Cybernetics).

Figure Legends

Figure 1. Subcellular localization of various human caspases. HeLa cells were transfected with GFP-tagged wild type caspases together with the caspase inhibitor p35 (caspase-6 and -7) or GFP-tagged caspases with the active site cysteine-to-serine (C/S) mutations (the other caspases). Representative fluorescent micorgraphs for each caspase are shown. Cells in h and i were counter-stained with DAPI to show nuclei (blue).

Figure 2. Colocalization of caspase-2 with PODs.

HeLa cells transfected with caspase-2-GFP were immunostained by a polyclonal anti-PML antibody followed by a secondary antibody conjugated with Texas Red. The paired fluorescence images of GFP and anti-PML and the merged imagine are shown. Similar results were observed when a monoclonal anti-PML antibody was used.

Figure 3. Requirement of both the prodomains and the protease domains but not the adapter proteins for the localization of caspase-2 in the PODs.

A. Both the prodomain and protease domain of caspase-2 are required for the POD localization. Top panels: Schematic representation of GFP tagged proteins. Bottom panels: HeLa cells were transfection with plasmids expressing indicated GFP proteins and representative patterns of the subcellular localization are shown.

B. Subcellular localization of GFP fusions of RAIDD.

C. Caspase-2 in the PODs is not accessible by the antibodies. HeLa cells were transfected with caspase-2- and immunostained with anti-caspase-2 (H19) followed by Texas-red conjugated secondary antibodies.

Figure 1

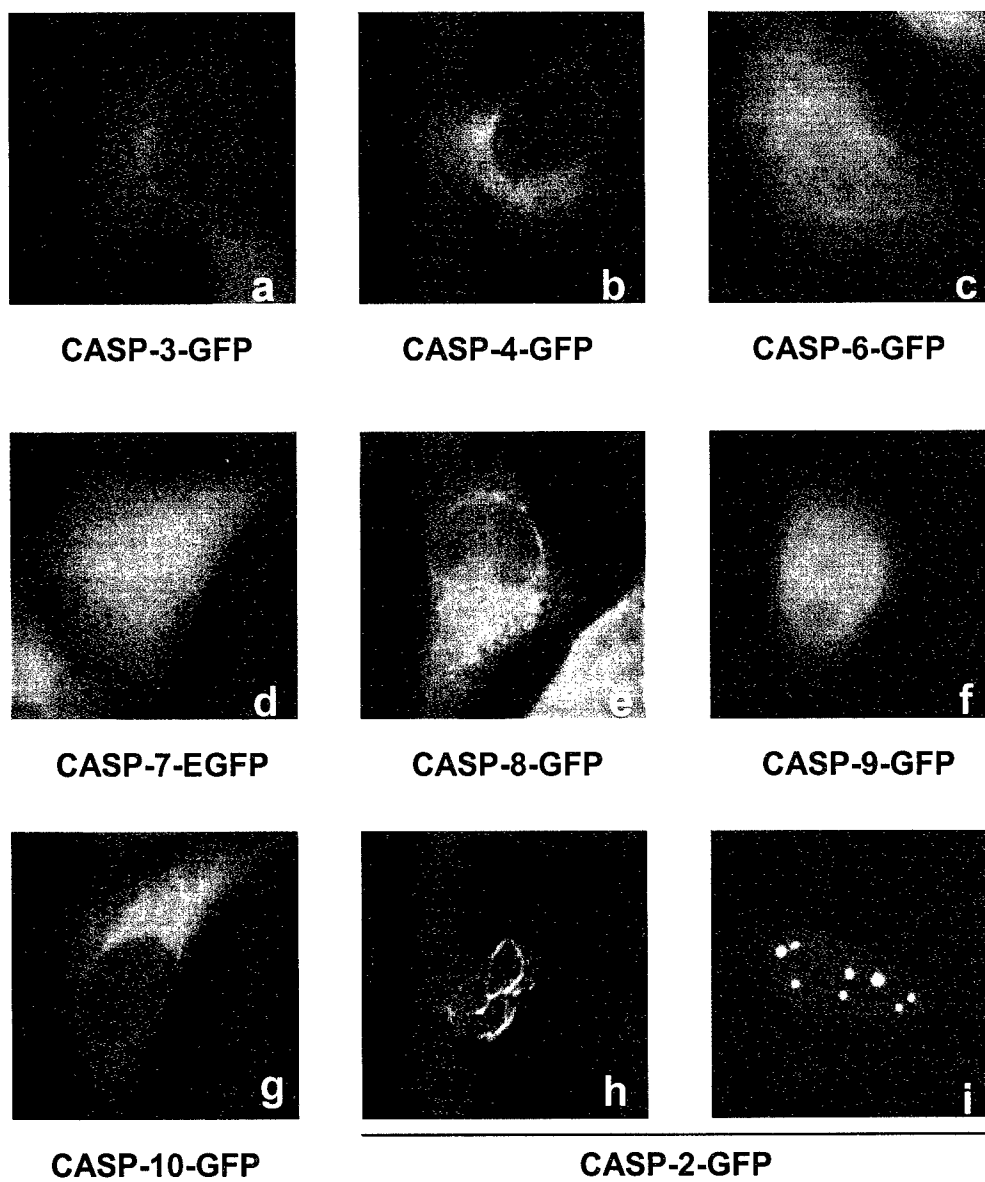


Figure 2

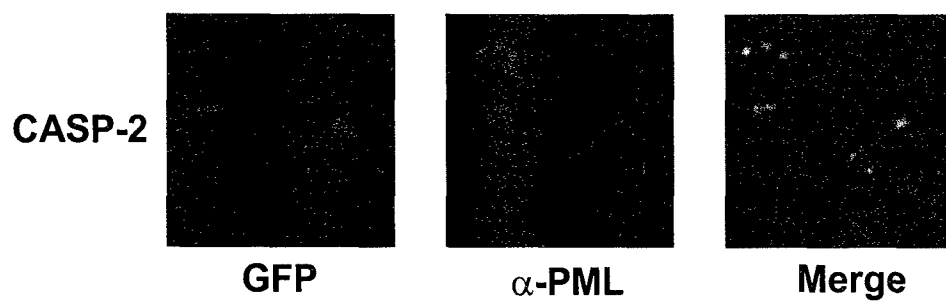
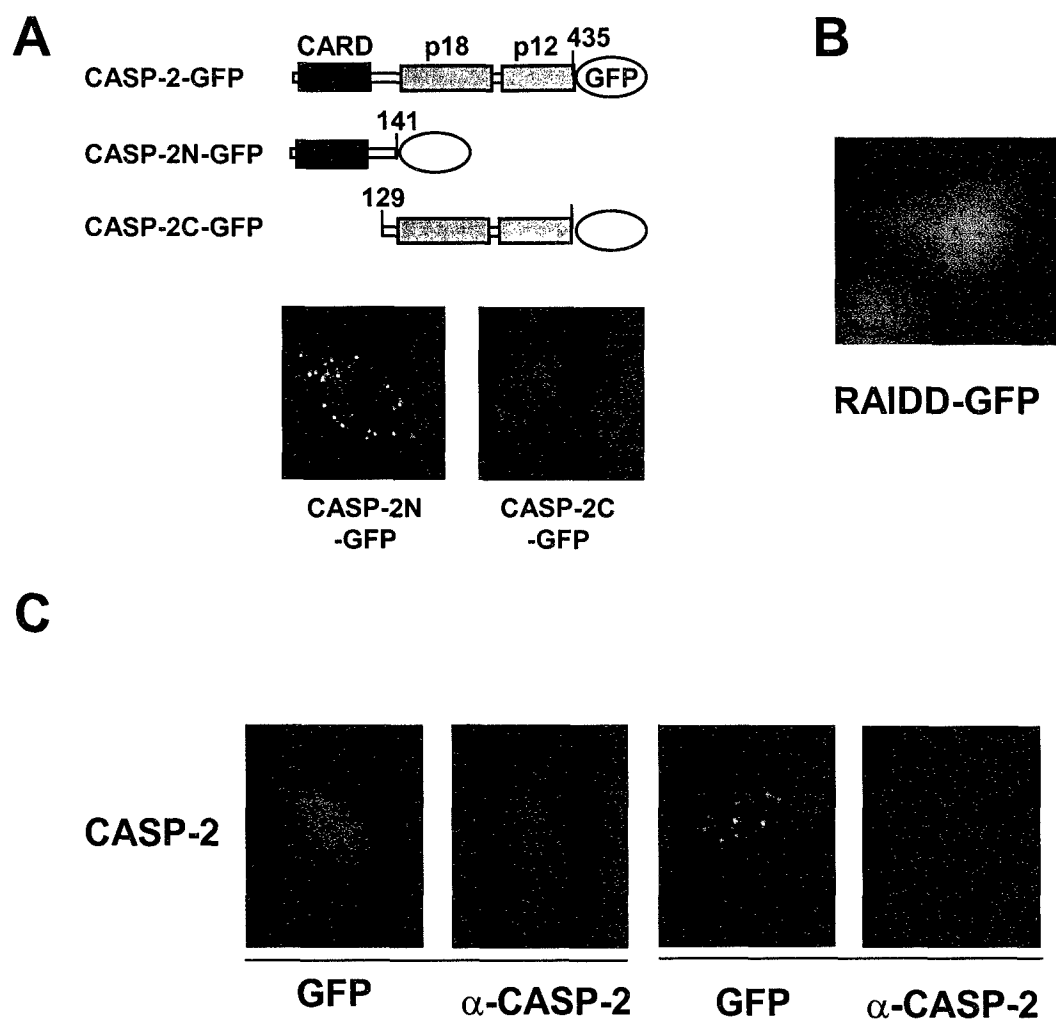


Figure 3



KEY RESEARCH COMPLISHMENTS

1. Discover co-localization of caspase-2 with PODs.
2. Determine the structural requirement for the co-localization.

REPORTABLE OUTCOME

A Manuscript is being prepared for submission.

CONCLUSION

Daxx is a pro-apoptotic protein that can be found in PML oncogenic domains (PODs). To determine the mechanism by which PODs and Daxx regulate apoptosis, we have examined the cellular localization of mammalian caspases, the executioner of apoptosis, using green fluorescence protein fusions. We found an initiator caspase, caspase-2, is localized in the PODs. The localization requires both the prodomain and protease domains of caspase-2 but is independent of its adapter protein RAIDD. These data suggest that PODs and Daxx might promote apoptosis through direct recruitment and activation of caspase-2.

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